

APPLICATION UNDER UNITED STATES PATENT LAWS

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Invention: NOVEL NUCLEOTIDE SEQUENCES ENCODING THE GPM GENE

Inventor (s): Bettina MÖCKEL
Walter PFEFFERLE

Pillsbury Madison & Sutro LLP
Intellectual Property Group
1100 New York Avenue, NW
Ninth Floor
Washington, DC 20005-3918
Attorneys
Telephone: (202) 861-3000

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SPECIFICATION

Novel nucleotide sequences encoding the gpm gene

This application claims priority from German Application No. 199 58 160.6, filed on December 2, 1999, the subject
5 matter of which is hereby incorporated herein by reference.

BACKGROUND OF THE INVENTION**1. Field of the Invention**

10 The invention provides nucleotide sequences encoding the gpm gene and fermentation processes for the preparation of amino acids, especially L-lysine, using corynebacteria in which the gpm gene is amplified.

15 2. Background Information

Amino acids, especially L-lysine, are used in human medicine and in the pharmaceutical industry, but especially in animal nutrition.

20 It is known that amino acids are prepared by the fermentation of strains of corynebacteria, especially *Corynebacterium glutamicum*. Because of its great importance, attempts are constantly being made to improve the preparative processes. Improvements to the processes
25 may relate to measures involving the fermentation technology, for example, stirring and oxygen supply, or the composition of the nutrient media, for example, the sugar concentration during fermentation, or the work-up to the product form, for example, by ion exchange
30 chromatography, or the intrinsic productivity characteristics of the microorganism itself.

The productivity characteristics of these microorganisms are improved by using methods of mutagenesis, selection

and mutant choice to give strains which are resistant to antimetabolites, for example, the lysine analogue S-(2-aminoethyl)cysteine, or auxotrophic for metabolites important in regulation, and produce L-lysine.

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Methods of recombinant DNA technology have also been used for some years to improve amino acid-producing strains of *Corynebacterium* by amplifying individual amino acid biosynthesis genes and studying the effect on amino acid production. Review articles on this subject have been published *inter alia* by Kinoshita ("Glutamic Acid Bacteria", in: *Biology of Industrial Microorganisms*, Demain and Solomon (Eds.), Benjamin Cummings, London, UK, 1985, 115-142), Hilliger (*BioTec* 2, 40-44 (1991)), Eggeling (*Amino Acids* 6, 261-272 (1994)), Jetten and Sinskey (*Critical Reviews in Biotechnology* 15, 73-103 (1995)) and Sahm et al. (*Annals of the New York Academy of Science* 782, 25-39 (1996)).

20 SUMMARY OF THE INVENTION

Object of the invention

It is an object of the invention to provide novel means for improving the preparation of amino acids, especially L-lysine, by fermentation.

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Description of the invention

Amino acids, especially L-lysine, are used in human medicine, in the pharmaceutical industry and particularly in animal nutrition. It is therefore of general interest to provide novel improved processes for the preparation of amino acids, especially L-lysine.

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When L-lysine or lysine is mentioned hereafter, it is understood as meaning not only the base but also the salts, for example, lysine monohydrochloride or lysine sulfate.

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The invention provides an isolated polynucleotide from corynebacteria which contains a polynucleotide sequence selected from the group comprising:

- 10 a) a polynucleotide which is at least 70% identical to a polynucleotide encoding a polypeptide containing the amino acid sequence of SEQ ID NO:2,
- b) a polynucleotide encoding a polypeptide containing an amino acid sequence which is at least 70% identical to
15 the amino acid sequence of SEQ ID NO:2,
- c) a polynucleotide which is complementary to the polynucleotides of a) or b), and
- 20 d) a polynucleotide containing at least 15 consecutive bases of the polynucleotide sequence of a), b) or c).

The invention also provides the polynucleotide with the
25 aforementioned features which is preferably a replicatable DNA containing:

- (i) the nucleotide sequence shown in SEQ ID NO:1, or
- 30 (ii) at least one sequence corresponding to sequence (i) within the degeneracy of the genetic code, or

(iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and optionally

5 (iv) neutral sense mutations in (i).

The invention also provides:

a polynucleotide with features a)-d) containing the
10 nucleotide sequence as shown in SEQ ID NO:1,

a polynucleotide with features a)-d) encoding a
polypeptide containing the amino acid sequence as shown
in SEQ ID NO:2,
15

a vector containing the polynucleotide described above,
especially shuttle vector or plasmid vector pXKgpmexp,
which is shown in Figure 2, and
20 corynebacteria, serving as host cells, which contain the
vector or in which the gpm gene is amplified.

The invention also provides polynucleotides consisting
substantially of a polynucleotide sequence which is
25 obtainable by screening, by means of hybridization, of an
appropriate gene library, containing the complete gene
with the polynucleotide sequence corresponding to SEQ ID
NO:1, with a probe containing the sequence of said
polynucleotide according to SEQ ID NO:1 or a fragment
30 thereof, and by isolation of said DNA sequence.

As hybridization probes for RNA, cDNA and DNA,
polynucleotide sequences according to the invention are
suitable for isolating the full length of cDNAs coding for

phosphoglycerate mutase, and for isolating cDNAs or genes whose sequence exhibits a high degree of similarity to the sequence of the phosphoglycerate mutase gene.

- 5 Polynucleotide sequences according to the invention are further suitable as primers for the preparation, by the polymerase chain reaction (PCR), of DNA of genes coding for phosphoglycerate mutase.
- 10 Such oligonucleotides serving as probes or primers contain at least 30, preferably at least 20 and very particularly preferably at least 15 consecutive bases. Oligonucleotides with a length of at least 40 or 50 base pairs are also suitable.
- 15 "Isolated" means separated from its natural environment.
- "Polynucleotide" refers in general to polyribonucleotides and polydeoxyribonucleotides, it being possible for the
- 20 RNAs or DNAs in question to be unmodified or modified.
- "Polypeptides" are understood as meaning peptides or proteins containing two or more amino acids bonded via peptide linkages.
- 25 The polypeptides according to the invention include a polypeptide according to SEQ ID NO:2, especially those with the biological activity of phosphoglycerate mutase and also those which are at least 70% identical to the
- 30 polypeptide according to SEQ ID NO:2, preferably at least 80% and particularly at least 90% to 95% identical to the polypeptide according to SEQ ID NO:2, and have said activity.

The invention further relates to a fermentation process for the preparation of amino acids, especially L-lysine, using corynebacteria which, in particular, already produce an amino acid and in which the nucleotide sequences
5 encoding the gpm gene are amplified and, in particular, overexpressed.

In this context the term "amplification" describes the increase in the intracellular activity, in a
10 microorganism, of one or more enzymes which are encoded by the appropriate DNA, for example by increasing the copy number of the gene(s), using a strong promoter or using a gene encoding an appropriate enzyme with a high activity, and optionally combining these measures.

15 The microorganisms provided by the present invention can produce L-amino acids, especially L-lysine, from glucose, sucrose, lactose, fructose, maltose, molasses, starch or cellulose or from glycerol and ethanol. Said
20 microorganisms can be representatives of corynebacteria, especially of the genus *Corynebacterium*. The species *Corynebacterium glutamicum* may be mentioned in particular in the genus *Corynebacterium*, being known to those skilled in the art for its ability to produce L-amino acids.

25 The following known wild-type strains:

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
30 *Corynebacterium acetoacidophilum* ATCC13870
Corynebacterium thermoaminogenes FERM BP-1539
Corynebacterium melassecola ATCC17965
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 and

Brevibacterium divaricatum ATCC14020

and L-lysine-producing mutants or strains prepared therefrom, such as:

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Corynebacterium glutamicum FERM-P 1709

Brevibacterium flavum FERM-P 1708

Brevibacterium lactofermentum FERM-P 1712

Corynebacterium glutamicum FERM-P 6463

10

Corynebacterium glutamicum FERM-P 6464 and

Corynebacterium glutamicum DSM5715

are examples of suitable strains of the genus *Corynebacterium*, especially of the species *Corynebacterium*
15 *glutamicum*.

The inventors have succeeded in isolating, from *C. glutamicum*, the novel *gpm* gene coding for the enzyme phosphoglycerate mutase (EC 5.4.2.1).

20

The first step in isolating the *gpm* gene or other genes from *C. glutamicum* is to construct a gene library of this microorganism in *E. coli*. The construction of gene libraries is documented in generally well-known textbooks
25 and handbooks. Examples which may be mentioned are the textbook by Winnacker entitled *From Genes to Clones*, Introduction to Gene Technology (Verlag Chemie, Weinheim, Germany, 1990) or the handbook by Sambrook et al. entitled *Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor
30 Laboratory Press, 1989). A very well-known gene library is that of the *E. coli* K-12 strain W3110, which was constructed by Kohara et al. (Cell 50, 495-508 (1987)) in λ vectors. Bathe et al. (*Molecular and General Genetics* 252, 255-265, 1996) describe a gene library of *C.*

glutamicum ATCC13032, which was constructed using cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA 84, 2160-2164) in the E. coli K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16, 1563-1575). Börmann et al. (Molecular Microbiology 6(3), 317-326) in turn describe a gene library of C. glutamicum ATCC13032 using cosmid pHC79 (Hohn and Collins, Gene 11, 291-298 (1980)). A gene library of C. glutamicum in E. coli can also be constructed using plasmids like pBR322 (Bolivar, Life Sciences 25, 807-818 (1979)) or pUC9 (Viera et al., 1982, Gene 19, 259-268). Restriction- and recombination-defective E. coli strains are particularly suitable as hosts, an example being the strain DH5 α mcr, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA 87 (1990) 4645-4649). The long DNA fragments cloned with the aid of cosmids can then in turn be subcloned into common vectors suitable for sequencing, and subsequently sequenced, e.g. as described by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America 74, 5463-5467, 1977).

The novel DNA sequence of C. glutamicum coding for the gpm gene, which as SEQ ID NO:1 forms part of the present invention, was obtained in this way. Furthermore, the amino acid sequence of the corresponding protein was derived from said DNA sequence by the methods described above. The resulting amino acid sequence of the gpm gene product is shown in SEQ ID NO:2.

Coding DNA sequences which result from SEQ ID NO:1 due to the degeneracy of the genetic code also form part of the invention. Likewise, DNA sequences which hybridize with

SEQ ID NO:1 or portions of SEQ ID NO:1 form part of the invention. Furthermore, conservative amino acid exchanges, for example, the exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are known to those skilled in the art as "sense mutations", which do not cause a fundamental change in activity of the protein, i.e. they are neutral. It is also known that changes at the N- and/or C-terminus of a protein do not substantially impair its function or may even stabilize it. Those skilled in the art will find information on this subject *inter alia* in Ben-Bassat et al. (Journal of Bacteriology 169, 751-757 (1987)), O'Regan et al. (Gene 77, 237-251 (1989)), Sahin-Toth et al. (Protein Sciences 3, 240-247 (1994)) and Hochuli et al. (Bio/Technology 6, 1321-1325 (1988)) and in well-known textbooks on genetics and molecular biology. Amino acid sequences which correspondingly result from SEQ ID NO:2 also form part of the invention.

Likewise, DNA sequences which hybridize with SEQ ID NO:1 or portions of SEQ ID NO:1 form part of the invention. Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers resulting from SEQ ID NO:1 form part of the invention. Such oligonucleotides typically have a length of at least 15 base pairs.

Those skilled in the art will find instructions on the identification of DNA sequences by means of hybridization in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology (1991) 41, 255-260), *inter alia*. Those skilled in the art will find instructions on

the amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) in the handbook by Gait entitled Oligonucleotide synthesis: a practical approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham:
5 PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994), inter alia.

The inventors have discovered that, after overexpression of the gpm gene, the production of amino acids, especially
10 L-lysine, by corynebacteria is improved.

Overexpression can be achieved by increasing the copy number of the appropriate genes or mutating the promoter and regulatory region or the ribosome binding site located
15 upstream from the structural gene. Expression cassettes incorporated upstream from the structural gene work in the same way. Inducible promoters additionally make it possible to increase the expression in the course of the production of L-lysine by fermentation. Measures for
20 prolonging the life of the mRNA also improve the expression. Furthermore, the enzyme activity is also enhanced by preventing the degradation of the enzyme protein. The genes or gene constructs can either be located in plasmids of variable copy number or integrated
25 and amplified in the chromosome. Alternatively, it is also possible to achieve overexpression of the genes in question by changing the composition of the media and the culture technique.

30 Those skilled in the art will find relevant instructions in Martin et al. (Bio/Technology 5, 137-146 (1987)), Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), Eikmanns et al. (Gene 102, 93-98 (1991)), European patent EP

0 472 869, US patent 4,601,893, Schwarzer and Pühler
(Bio/Technology 9, 84-87 (1991)), Reinscheid et al.
(Applied and Environmental Microbiology 60, 126-132
(1994)), LaBarre et al. (Journal of Bacteriology 175,
5 1001-1007 (1993)), patent application WO 96/15246,
Malumbres et al. (Gene 134, 15-24 (1993)), Japanese
Offenlegungsschrift JP-A-10-229891, Jensen and Hammer
(Biotechnology and Bioengineering 58, 191-195 (1998)) and
Makrides (Microbiological Reviews 60, 512-538 (1996)) and
10 in well-known textbooks on genetics and molecular biology,
inter alia. For example, the *gpm* gene according to the
invention has been overexpressed with the aid of plasmids.

Suitable plasmids are those which are replicated in
15 corynebacteria. Numerous known plasmid vectors, for
example, pZ1 (Menkel et al., Applied and Environmental
Microbiology (1989) 64, 549-554), pEKEx1 (Eikmanns et al.,
Gene 102, 93-98 (1991)) or pHS2-1 (Sonnen et al., Gene
107, 69-74 (1991)), are based on cryptic plasmids pHM1519,
20 pBL1 or pGA1. Other plasmid vectors, for example, those
based on pCG4 (US-A-4,489,160), pNG2 (Serwold-Davis et
al., FEMS Microbiology Letters 66, 119-124 (1990)) or pAG1
(US-A-5,158,891), can be used in the same way.

25 An example of a plasmid which can be used for
overexpression of the *gpm* gene is *E. coli*/C. glutamicum
shuttle expression vector pXKgpmexp. The vector contains
the replication region *rep* of plasmid pGA1, including the
replication effector *per* (US-A-5,175,108; Nesvera et al.,
30 Journal of Bacteriology 179, 1525-1532 (1997)), the
kanamycin resistance gene *aph*(3')-IIa from *Escherichia*
coli, the origin of replication, the *trc* promoter, the
termination regions T1 and T2, the *lacI^q* gene (repressor of
the *lac* operon of *E. coli*) and a multiple cloning site *mcs*

(Norrander, J.M. et al., Gene 26, 101-106 (1983)) of plasmid pTRC99A (Amann et al. (1988), Gene 69, 301-315).

Shuttle expression vector pXKgpmexp is shown in Figure 2.

5

In addition it can be advantageous for the production of amino acids, especially L-lysine, to overexpress not only the gpm gene but also one or more enzymes of the particular biosynthetic pathway, the glycolysis, the
10 anaplerosis, the citric acid cycle or the amino acid export.

Thus, for example, the following can be overexpressed for the preparation of L-lysine:

15

- simultaneously the dapA gene coding for dihydrodipicolinate synthase (EP-B-0 197 335), or
- simultaneously the gap gene coding for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of
20 Bacteriology 174, 6076-6086), or
- simultaneously the tpi gene coding for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174,
25 6076-6086), or
- simultaneously the pgk gene coding for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174, 6076-6086), or
- simultaneously the pyc gene coding for pyruvate
30 carboxylase (Eikmanns (1992), Journal of Bacteriology 174, 6076-6086), or

- simultaneously the lysE gene coding for lysine export (DE-A-195 48 222), or
- 5 • simultaneously the mqo gene coding for malate quinone oxidoreductase (Molenaar et al. (1998), European Journal of Biochemistry 254, 395-403), or
- the zwal gene (DE 199 59 328.0, DSM13115).

10

In addition to amplification of the gpm gene, it can also be advantageous for the production of amino acids, especially L-lysine, simultaneously to attenuate the following:

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- the pck gene coding for phosphoenol pyruvate carboxykinase (DE 199 50 409.1, DSM13047), or
- the pgi gene coding for glucose 6-phosphate isomerase
- 20 (US 09/396,478, DSM12969), or
- the poxB gene coding for pyruvate oxidase (DE 199 51 975.7, DSM13114), or

- 25 • the zwa2 gene (DE 199 59 327.2, DSM13113).

It can also be advantageous for the production of amino acids, especially L-lysine, not only to overexpress the gpm gene but also to switch off undesirable secondary

30 reactions (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms prepared according to the invention can be cultivated for the production of amino acids, especially L-lysine, continuously or discontinuously by the batch process, the fed batch process or the repeated fed batch process. A summary of known cultivation methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Bioprocess Technology 1. Introduction to Bioengineering) (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Bioreactors and Peripheral Equipment) (Vieweg Verlag, Brunswick/Wiesbaden, 1994)).

The culture medium to be used must appropriately meet the demands of the particular strains. Descriptions of culture media for various microorganisms can be found in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington DC, USA, 1981). Carbon sources which can be used are sugars and carbohydrates, for example, glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, for example, soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, for example, palmitic acid, stearic acid and linoleic acid, alcohols, for example, glycerol and ethanol, and organic acids, for example, acetic acid. These substances can be used individually or as a mixture. Nitrogen sources which can be used are organic nitrogen-containing compounds such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources can be used individually or

as a mixture. Phosphorus sources which can be used are phosphoric acid, potassium dihydrogenphosphate or dipotassium hydrogenphosphate or the corresponding sodium salts. The culture medium must also contain metal salts, for example, magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth-promoting substances such as amino acids and vitamins can be used in addition to the substances mentioned above. Suitable precursors can also be added to the culture medium. Said feed materials can be added to the culture all at once or fed in appropriately during cultivation.

The pH of the culture is controlled by the appropriate use of basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds such as phosphoric acid or sulfuric acid. Foaming can be controlled using antifoams such as fatty acid polyglycol esters. The stability of plasmids can be maintained by adding suitable selectively acting substances, for example, antibiotics, to the medium. Aerobic conditions are maintained by introducing oxygen or oxygen-containing gaseous mixtures, for example, air, into the culture. The temperature of the culture is normally 20°C to 45°C and preferably 25°C to 40°C. The culture is continued until L-lysine formation has reached a maximum. This objective is normally achieved within 10 hours to 160 hours.

L-lysine can be analyzed by anion exchange chromatography with subsequent ninhydrin derivatization, as described by Spackman et al. (Analytical Chemistry 30 (1958) 1190).

The following microorganisms were deposited in the Deutsche Sammlung für Mikroorganismen und Zellkulturen

(German Collection of Microorganisms and Cell Cultures (DSMZ), Brunswick, Germany) on 17.04.2000 under the terms of the Budapest Treaty:

- 5 • *Corynebacterium glutamicum* strain DSM5715/pXKgpmexp as DSM13456
- *Corynebacterium glutamicum* strain DSM5715/pEC-XK as DSM13455

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The fermentation process according to the invention is used for the preparation of amino acids, especially L-lysine.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1: Map of plasmid pEC-XK99E

5 Figure 2: Map of plasmid pXKgpmexp

The abbreviations and symbols used are defined as follows:

per: copy number control gene from pGA1
10 oriE: plasmid-coded origin of replication from E. coli
rep: plasmid-coded origin of replication from C.
glutamicum plasmid pGA1
15 Ptrc: trc promoter from pTRC99A
T1, T2: terminator regions 1 and 2 from pTRC99A
20 lacIq: repressor gene of the lac operon
Kan: kanamycin resistance gene
gpm: gpm gene from C. glutamicum
25 EcoRI: cleavage site of the restriction enzyme EcoRI
Ecl136II: cleavage site of the restriction enzyme Ecl136II
30 HindIII: cleavage site of the restriction enzyme HindIII
KpnI: cleavage site of the restriction enzyme KpnI
SalI: cleavage site of the restriction enzyme SalI

SmaI: cleavage site of the restriction enzyme SmaI

PstI: cleavage site of the restriction enzyme PstI

5 BamHI: cleavage site of the restriction enzyme BamHI

NcoI: cleavage site of the restriction enzyme NcoI

10 XbaI: cleavage site of the restriction enzyme XbaI

XmaI: cleavage site of the restriction enzyme XmaI

15 SacI: cleavage site of the restriction enzyme SacI

DETAILED DESCRIPTION OF THE INVENTION

The present invention is illustrated in greater detail below with the aid of Examples.

20

Example 1

Preparation of a genomic cosmid gene library from
Corynebacterium glutamicum ATCC13032

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Chromosomal DNA from *Corynebacterium glutamicum* ATCC13032 was isolated as described by Tauch et al. (1995, Plasmid 33, 168-179) and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, product description SAP, code no. 1758250). The DNA of cosmid vector SuperCos1 (Wahl et al. (1987)

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Proceedings of the National Academy of Sciences USA 84, 2160-2164), obtained from Stratagene (La Jolla, USA, product description SuperCos1 Cosmid Vector Kit, code no. 251301), was cleaved with the restriction enzyme XbaI
5 (Amersham Pharmacia, Freiburg, Germany, product description XbaI, code no. 27-0948-02) and also dephosphorylated with shrimp alkaline phosphatase. The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product
10 description BamHI, code no. 27-0868-04). The cosmid DNA treated in this way was mixed with the treated ATCC13032 DNA and the mixture was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, product description T4 DNA ligase, code no. 27-0870-04). The
15 ligation mixture was then packaged into phages using Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, product description Gigapack II XL Packing Extract, code no. 200217). For infection of the E. coli strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16, 1563-
20 1575), the cells were taken up in 10 mM MgSO₄ and mixed with an aliquot of the phage suspension. Infection and titering of the cosmid library were carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the cells being
25 plated on LB agar (Lennox, 1955, Virology 1, 190) containing 100 mg/l of ampicillin. After incubation overnight at 37°C, recombinant single clones were selected.

Example 2

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Isolation and sequencing of the gpm gene

The cosmid DNA of a single colony was isolated with the Qiaprep Spin Miniprep Kit (product no. 27106, Qiagen,

Hilden, Germany) in accordance with the manufacturer's instructions and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, product no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, product description SAP, product no. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range from 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (product no. 20021, Qiagen, Hilden, Germany). The DNA of sequencing vector pZero-1, obtained from Invitrogen (Groningen, The Netherlands, product description Zero Background Cloning Kit, product no. K2500-01), was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI, product no. 27-0868-04). Ligation of the cosmid fragments into sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then introduced into the E. coli strain DH5 α MCR (Grant, 1990, Proceedings of the National Academy of Sciences USA 87, 4645-4649) by electroporation (Tauch et al. 1994, FEMS Microbiol. Letters 123, 343-7) and plated on LB agar (Lennox, 1955, Virology 1, 190) containing 50 mg/l of zeocin. Plasmid preparation of the recombinant clones was carried out with Biorobot 9600 (product no. 900200, Qiagen, Hilden, Germany). Sequencing was carried out by the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academy of Sciences USA 74, 5463-5467) with modifications by Zimmermann et al. (1990, Nucleic Acids Research 18, 1067). The "RR dRhodamine Terminator Cycle Sequencing Kit" from

PE Applied Biosystems (product no. 403044, Weiterstadt, Germany) was used. Separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphorese NF acrylamide/bisacrylamide" gel (29:1) (product no. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained were then processed using the Staden programming package (1986, Nucleic Acids Research 14, 217-231), version 97-0. The individual sequences of the pZero-1 derivatives were assembled into a cohesive contig. Computer-assisted coding region analysis was performed with the XNIP program (Staden, 1986, Nucleic Acids Research 14, 217-231). Further analyses were performed with the "BLAST search programs" (Altschul et al., 1997, Nucleic Acids Research 25, 3389-3402) against the non-redundant data bank of the "National Center for Biotechnology Information" (NCBI, Bethesda, MD, USA).

The nucleotide sequence obtained is shown in SEQ ID NO:1. Analysis of the nucleotide sequence gave an open reading frame of 744 base pairs, which was called the gpm gene. The gpm gene codes for a protein of 248 amino acids.

Example 3

Preparation of shuttle expression vector pXKgmpepx for amplification of the gpm gene in *C. glutamicum*

3.1. Cloning of the gpm gene

Chromosomal DNA was isolated from the strain ATCC13032 by the method of Eikmanns et al. (Microbiology 140, 1817-1828

(1994)). On the basis of the sequence of the gpm gene known for *C. glutamicum* from Example 2, the following oligonucleotides were chosen for the polymerase chain reaction:

5

Gpm (ex1.1):

5' TAA AGT GGC AAA CTA GTACC 3'

Gpm (ex2):

5' CTA CTT ATT ACC CTG GTT T 3'

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The primers shown were synthesized by ARK Scientific GmbH Biosystems (Darmstadt, Germany) and PCR was carried out by the standard PCR method of Innis et al. (PCR protocols. A guide to methods and applications, 1990, Academic Press) with Pwo polymerase from Roche Diagnostics GmbH (Mannheim, Germany). Using the polymerase chain reaction, the primers allow the amplification of an approx. 0.77 kb DNA fragment carrying the gpm gene.

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The approx. 0.77 kb gpm fragment was isolated from the agarose gel with the QiaExII Gel Extraction Kit (product no. 20021, Qiagen, Hilden, Germany).

3.2. Construction of shuttle vector pEC-XK99E

25

E. coli/*C. glutamicum* shuttle vector pEC-XK99E was constructed according to the state of the art. The vector contains the replication region rep of plasmid pGA1, including the replication effector per (US-A-5,175,108; Nesvera et al., Journal of Bacteriology 179, 1525-1532 (1997)), the kanamycin resistance gene aph(3')-IIa from *Escherichia coli* (Beck et al. (1982), Gene 19, 327-336), the origin of replication, the trc promoter, the

30

termination regions T1 and T2, the *lacI^q* gene (repressor of the *lac* operon of *E. coli*) and a multiple cloning site *mcs* (Norranders, J.M. et al., *Gene* 26, 101-106 (1983)) of plasmid pTRC99A (Amann et al. (1988), *Gene* 69, 301-315).

5

The constructed *E. coli*/*C. glutamicum* shuttle vector pEC-XK99E was transferred to *C. glutamicum* DSM5715 by electroporation (Liebl et al., 1989, *FEMS Microbiology Letters* 53, 299-303). The transformants were selected on LBHIS agar consisting of 18.5 g/l of brain-heart infusion broth, 0.5 M sorbitol, 5 g/l of bacto tryptone, 2.5 g/l of bacto yeast extract, 5 g/l of NaCl and 18 g/l of bacto agar, which had been supplemented with 25 mg/l of kanamycin. Incubation was carried out for 2 days at 33°C.

15

Plasmid DNA was isolated from one transformant by the conventional methods (Peters-Wendisch et al., 1998, *Microbiology* 144, 915-927) and cleaved with the restriction endonuclease HindIII, and the plasmid was checked by subsequent agarose gel electrophoresis.

20

The resulting plasmid construct was called pEC-XK99E (Figure 1). The strain obtained by the introduction of plasmid pEC-XK99E into the *C. glutamicum* strain DSM5715 by electroporation was called DSM5715/pEC-XK99E and deposited as DSM13455 in the German Collection of Microorganisms and Cell Cultures (DSMZ, Brunswick, Germany) under the terms of the Budapest Treaty.

25

30 3.3. Cloning of *gpm* in *E. coli*/*C. glutamicum* shuttle vector pEC-XK99E

The vector used was the *E. coli*/*C. glutamicum* shuttle vector pEC-XK99E described in Example 3.2. DNA of this

plasmid was fully cleaved with the restriction enzyme
Ecl136II and then dephosphorylated with shrimp alkaline
phosphatase (Roche Diagnostics GmbH, Mannheim, Germany,
product description SAP, product no. 1758250).

5

The 0.77 kb gpm fragment described in Example 3.1,
obtained by means of PCR, was mixed with the prepared
vector pEC-XK99E and the mixture was treated with T4 DNA
ligase (Amersham Pharmacia, Freiburg, Germany, product
10 description T4 DNA ligase, code no. 27-0870-04). The
ligation mixture was transformed to the E. coli strain
DH5 α (Hanahan, in: DNA cloning. A practical approach.
Vol. I. IRL-Press, Oxford, Washington DC, USA). Plasmid-
carrying cells were selected by plating the transformation
15 mixture on LB agar (Lennox, 1955, Virology 1, 190)
containing 25 mg/l of kanamycin. After incubation
overnight at 37°C, recombinant single clones were selected.
Plasmid DNA was isolated from one transformant with the
Qiaprep Spin Miniprep Kit (product no. 27106, Qiagen,
20 Hilden, Germany) in accordance with the manufacturer's
instructions and cleaved with the restriction enzymes
EcoRI and XbaI in order to check the plasmid by subsequent
agarose gel electrophoresis. The plasmid obtained was
called pXKgpmexp. It is shown in Figure 2.

25

Example 4

Transformation of the strain DSM5715 with plasmid
pXKgpmexp

30

The strain DSM5715 was transformed with plasmid pXKgpmexp
using the electroporation method described by Liebl et al.
(FEMS Microbiology Letters 53, 299-303 (1989)). The

transformants were selected on LBHIS agar consisting of 18.5 g/l of brain-heart infusion broth, 0.5 M sorbitol, 5 g/l of bacto tryptone, 2.5 g/l of bacto yeast extract, 5 g/l of NaCl and 18 g/l of bacto agar, which had been
5 supplemented with 25 mg/l of kanamycin. Incubation was carried out for 2 days at 33°C.

Plasmid DNA was isolated from one transformant by the conventional methods (Peters-Wendisch et al., 1998,
10 Microbiology 144, 915-927) and cleaved with the restriction endonucleases EcoRI and XbaI, and the plasmid was checked by subsequent agarose gel electrophoresis. The strain obtained was called DSM5715/pXKgpmexp.

15 The following microorganism was deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ, Brunswick, Germany) under the terms of the Budapest Treaty:

- 20 • *Corynebacterium glutamicum* strain DSM5715/pXKgpmexp as DSM13456

Example 5

25 Preparation of lysine

The *C. glutamicum* strain DSM5715/pXKgpmexp obtained in Example 4 was cultivated in a nutrient medium suitable for lysine production and the lysine content of the culture
30 supernatant was determined.

This was done by first incubating the strain on an agar plate with the appropriate antibiotic (brain-heart agar

containing kanamycin (25 mg/l)) for 24 hours at 33°C. This agar plate culture was used to inoculate a preculture (10 ml of medium in a 100 ml conical flask). Complete medium CgIII was used as the preculture medium.

5

Medium CgIII

	NaCl	2.5 g/l
	Bacto peptone	10 g/l
	Bacto yeast extract	10 g/l
10	Glucose (autoclaved separately)	2% (w/v)

The pH was adjusted to 7.4.

Kanamycin (25 mg/l) was added. The preculture was incubated for 16 hours at 33°C on a shaker at 240 rpm.

- This preculture was used to inoculate a main culture to give the latter an initial OD (660 nm) of 0.1. Medium MM was used for the main culture.

Medium MM

	CSL (corn steep liquor)	5 g/l
20	MOPS (morpholinopropanesulfonic acid)	20 g/l
	Glucose (separately autoclaved)	50 g/l
	(NH ₄) ₂ SO ₄	25 g/l
	KH ₂ PO ₄	0.1 g/l
	MgSO ₄ ·7H ₂ O	1.0 g/l
25	CaCl ₂ ·2H ₂ O	10 mg/l
	FeSO ₄ ·7H ₂ O	10 mg/l
	MnSO ₄ ·H ₂ O	5.0 mg/l

Biotin (sterile-filtered) 0.3 mg/l
Thiamine·HCl (sterile-filtered) 0.2 mg/l
L-leucine (sterile-filtered) 0.1 g/l
CaCO₃ 25 g/l

5

CSL, MOPS and the salt solution were adjusted to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions and the dry-autoclaved CaCO₃ were then added.

10

Cultivation is carried out in a volume of 10 ml in a 100 ml conical flask with baffles. Kanamycin (25 mg/l) was added. Cultivation proceeded at 33°C and 80% atmospheric humidity.

15

After 72 hours the OD was measured at a wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by means of ion exchange chromatography and postcolumn derivatization with ninhydrin detection.

20

The experimental result is shown in Table 1.

25

Table 1

Strain	OD (660)	Lysine·HCl g/l
DSM5715	6.8	13.68
DSM5715/pXKgp _{mexp}	7.3	14.35

SEQUENCE LISTING

<110> Degussa-Hüls AG

5 <120> Novel nucleotide sequences encoding the gpm gene

<130> 990168 BT

<140>

10 <141>

<160> 2

<170> PatentIn Ver. 2.1

15

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<212> DNA

<213> Corynebacterium glutamicum

20

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<221> CDS

<222> (181)..(924)

25

<400> 1

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accgccgcac agctatcgctc gctgtacaac gacgctattg ccaacgaaaa tgtcgacgggt 120

30

gaaacgcacac acgggctaagt aaacgcgcgt cgtggaacat aaagtggcaa actagtagcct 180

atg	act	aac	gga	aaa	ttg	att	ctt	ctt	cgt	cac	ggt	cag	agc	gaa	tgg	228
Met	Thr	Asn	Gly	Lys	Leu	Ile	Leu	Leu	Arg	His	Gly	Gln	Ser	Glu	Trp	
1				5					10					15		

35

aac	gca	tcc	aac	cag	ttc	act	gga	tgg	gtc	gac	gtc	aat	ctg	acc	gaa	276
Asn	Ala	Ser	Asn	Gln	Phe	Thr	Gly	Trp	Val	Asp	Val	Asn	Leu	Thr	Glu	
			20					25					30			

40

cag	ggt	gag	gct	gag	gcc	aag	cgc	gga	ggc	gaa	ctc	ctc	gtc	gag	gca	324
Gln	Gly	Glu	Ala	Glu	Ala	Lys	Arg	Gly	Gly	Glu	Leu	Leu	Val	Glu	Ala	
		35					40					45				

45

ggc	gtc	ctc	cca	ggc	gtt	gta	tac	acc	tcc	ttg	ctg	cgt	cgc	gcg	atc	372
Gly	Val	Leu	Pro	Gly	Val	Val	Tyr	Thr	Ser	Leu	Leu	Arg	Arg	Ala	Ile	
	50				55						60					

50

cgc	act	gca	aac	atc	gca	ctg	aac	gct	gca	gac	cgc	cac	tgg	atc	cca	420
Arg	Thr	Ala	Asn	Ile	Ala	Leu	Asn	Ala	Ala	Asp	Arg	His	Trp	Ile	Pro	
65				70					75					80		

55

gtg	atc	cgc	gac	tgg	cgc	ctc	aac	gag	cgt	cac	tac	ggc	gca	ctg	cag	468
Val	Ile	Arg	Asp	Trp	Arg	Leu	Asn	Glu	Arg	His	Tyr	Gly	Ala	Leu	Gln	
			85					90					95			

ggc	ctt	gac	aag	gct	gca	acc	aag	gaa	aaa	tac	ggc	gac	gac	cag	ttc	516
Gly	Leu	Asp	Lys	Ala	Ala	Thr	Lys	Glu	Lys	Tyr	Gly	Asp	Asp	Gln	Phe	
			100					105					110			

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 Met Glu Trp Arg Arg Ser Tyr Asp Thr Pro Pro Pro Glu Leu Ala Asp
 115 120 125

5 gac gca gag tac tcc cag gca aat gac cct cgt tac gcg gac ctc gac 612
 Asp Ala Glu Tyr Ser Gln Ala Asn Asp Pro Arg Tyr Ala Asp Leu Asp
 130 135 140

10 gta gtt cca cgc acc gaa tgc ctc aag gac gtt gtg gtt cgt ttt gtt 660
 Val Val Pro Arg Thr Glu Cys Leu Lys Asp Val Val Val Arg Phe Val
 145 150 155 160

15 cct tac ttc gag gaa gaa atc ctg cca cgc gca aag aag ggc gaa acc 708
 Pro Tyr Phe Glu Glu Glu Ile Leu Pro Arg Ala Lys Lys Gly Glu Thr
 165 170 175

20 gtc ctc atc gca gca cac ggc aac tcc ctg cgt gcg ctg gtt aag cac 756
 Val Leu Ile Ala Ala His Gly Asn Ser Leu Arg Ala Leu Val Lys His
 180 185 190

ctt gac ggc atc tcc gat gct gat atc gca gag ctc aac atc cca acc 804
 Leu Asp Gly Ile Ser Asp Ala Asp Ile Ala Glu Leu Asn Ile Pro Thr
 195 200 205

25 ggc atc cca ctg gtc tac gaa atc gcc gaa gac ggt tcc gta gta aac 852
 Gly Ile Pro Leu Val Tyr Glu Ile Ala Glu Asp Gly Ser Val Val Asn
 210 215 220

30 cca ggc ggc acc tac ctc gat cct gag gca gca gca gcc ggc gca gca 900
 Pro Gly Gly Thr Tyr Leu Asp Pro Glu Ala Ala Ala Ala Gly Ala Ala
 225 230 235 240

35 gca gta gca aac cag ggt aat aag tagctatttg taggtgagca ctcttcttgc 954
 Ala Val Ala Asn Gln Gly Asn Lys
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40 tcggat

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45 <213> Corynebacterium glutamicum

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55 Gln Gly Glu Ala Glu Ala Lys Arg Gly Gly Glu Leu Leu Val Glu Ala
 35 40 45

Gly Val Leu Pro Gly Val Val Tyr Thr Ser Leu Leu Arg Arg Ala Ile
 50 55 60

	Arg 65	Thr	Ala	Asn	Ile	Ala 70	Leu	Asn	Ala	Ala	Asp 75	Arg	His	Trp	Ile	Pro 80
5	Val	Ile	Arg	Asp	Trp 85	Arg	Leu	Asn	Glu	Arg 90	His	Tyr	Gly	Ala	Leu 95	Gln
	Gly	Leu	Asp	Lys 100	Ala	Ala	Thr	Lys	Glu 105	Lys	Tyr	Gly	Asp	Asp	Gln	Phe
10	Met	Glu	Trp 115	Arg	Arg	Ser	Tyr	Asp 120	Thr	Pro	Pro	Pro	Glu 125	Leu	Ala	Asp
	Asp	Ala 130	Glu	Tyr	Ser	Gln	Ala 135	Asn	Asp	Pro	Arg	Tyr 140	Ala	Asp	Leu	Asp
15	Val 145	Val	Pro	Arg	Thr	Glu 150	Cys	Leu	Lys	Asp 155	Val	Val	Val	Arg	Phe	Val 160
20	Pro	Tyr	Phe	Glu	Glu 165	Glu	Ile	Leu	Pro	Arg 170	Ala	Lys	Lys	Gly	Glu 175	Thr
	Val	Leu	Ile	Ala 180	Ala	His	Gly	Asn 185	Ser	Leu	Arg	Ala	Leu	Val 190	Lys	His
25	Leu	Asp	Gly 195	Ile	Ser	Asp	Ala	Asp 200	Ile	Ala	Glu	Leu	Asn 205	Ile	Pro	Thr
	Gly	Ile 210	Pro	Leu	Val	Tyr	Glu 215	Ile	Ala	Glu	Asp	Gly 220	Ser	Val	Val	Asn
30	Pro 225	Gly	Gly	Thr	Tyr	Leu 230	Asp	Pro	Glu	Ala	Ala 235	Ala	Ala	Gly	Ala	Ala 240
35	Ala	Val	Ala	Asn	Gln 245	Gly	Asn	Lys								